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Use of microalgae *Chlamydomonas reinhardtii* for production of double-stranded RNA against shrimp virus

Parinyachat Somchai[a,b], Sarocha Jitrakorn[a,d], Siripong Thitamadee[a,b], Metha Meetam[c], Vanvimon Saksmerprome[a,d,*]

[a] *Center of Excellence for Shrimp Molecular Biology and Biotechnology, Mahidol University, Bangkok 10400, Thailand*
[b] *Department of Biotechnology, Faculty of Science, Mahidol University, Bangkok 10400, Thailand*
[c] *Department of Biology, Faculty of Science, Mahidol University, Bangkok 10400, Thailand*
[d] *National Center for Genetic Engineering and Biotechnology (BIOTEC), Thailand Science Park, Pathumthani 12120, Thailand*

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ABSTRACT

RNA interference has been proposed to be a promising tool for combating shrimp viruses. Antiviral double-stranded (ds)RNA has been mostly produced in *Escherichia coli*-expression system because of its high efficiency and inexpensive operations. However, overusing the bacteria may raise concerns regarding public health and environmental contamination, and seeking for a new dsRNA production platform would be alternative for future molecular farming. In this study, we exploited the green microalgae *Chlamydomonas reinhardtii* to produce dsRNA targeting the lethal shrimp yellow head virus (YHV). The expression plasmid pSL18 for *C. reinhardtii* was constructed to contain YHV-specific hairpin RNA expression cassette, and the successful assembly of pSL18-YHV was confirmed by PCR and enzymatic digestions. Glass bead method was employed for transformation of *C. reinhardtii* nuclear genome with pSL18-YHV. Microalgal expression of dsRNA-YHV, approximately 45 ng from 100-mL culture, was detected by qRT-PCR. Oral feeding experiment on postlarval shrimp revealed that the formulated feed with *C. reinhardtii* expressing dsRNA-YHV, at the ratio of 1×10^8 transformants per gram feed, improved 22% survival rate after YHV challenge. The present study suggests that *C. reinhardtii* can be bioengineered to produce viral-specific dsRNA for shrimp viral disease control, and the developed qRT-PCR could detect microalgal dsRNA with detection limit of subpicogram.

1. Introduction

RNA interference (RNAi) is a specific and effective approach for inhibiting viral infections by introducing double-stranded (ds)RNA to interfere with viral mRNAs (Fire et al., 1998). In the past decade, RNAi-based technology has gained attention for its potential to control shrimp viral diseases that have caused significant losses in aquaculture. Inhibition of shrimp viruses by viral-specific dsRNA were demonstrated in insect cell lines (He et al., 2009; Theerawanitchpan et al., 2012) and in penaeid shrimp (Robalino et al., 2005; Yodmuang et al., 2006; Ongvarrasopone et al., 2008; Saksmerprome et al., 2009, 2013; Thammasorn et al., 2013). Given that sequences of viral genes of interest are available, dsRNA targeting those genes can be synthesized by *in vitro* transcription and *in vivo* bacterial system. For large-scale production, the RNase-deficient *Escherichia coli* (*E. coli*) strain transformed with vectors encoding gene-specific dsRNA is proved to be efficient and inexpensive (Saksmerprome et al., 2009). Nevertheless, constant use of the *E. coli* in shrimp has yet to be assessed for its effect on animals and human health.

Alternative to the current use of *E. coli* expression system, we explored the potential of the eukaryotic green microalgae *Chlamydomonas reinhardtii* for shrimp antiviral dsRNA production. First, considered as "Generally Regarding As Safe (GRAS)" by the US Food and Drug Administration, the microalgae does not produce any endotoxins and infectious agents, thus it should not raise any health risk or environmental contamination (Dauvillee et al., 2010). Moreover, genetic engineering of Chlamydomonas, through either random integration in nuclear genome or homologous recombination in chloroplast genome, has been

* Corresponding author at: Center of Excellence for Shrimp Molecular Biology and Biotechnology, BIOTEC, Mahidol University, Bangkok 10400, Thailand.
E-mail addresses: vsaksmer@gmail.com, vanvimon.sak@biotec.or.th (V. Saksmerprome).

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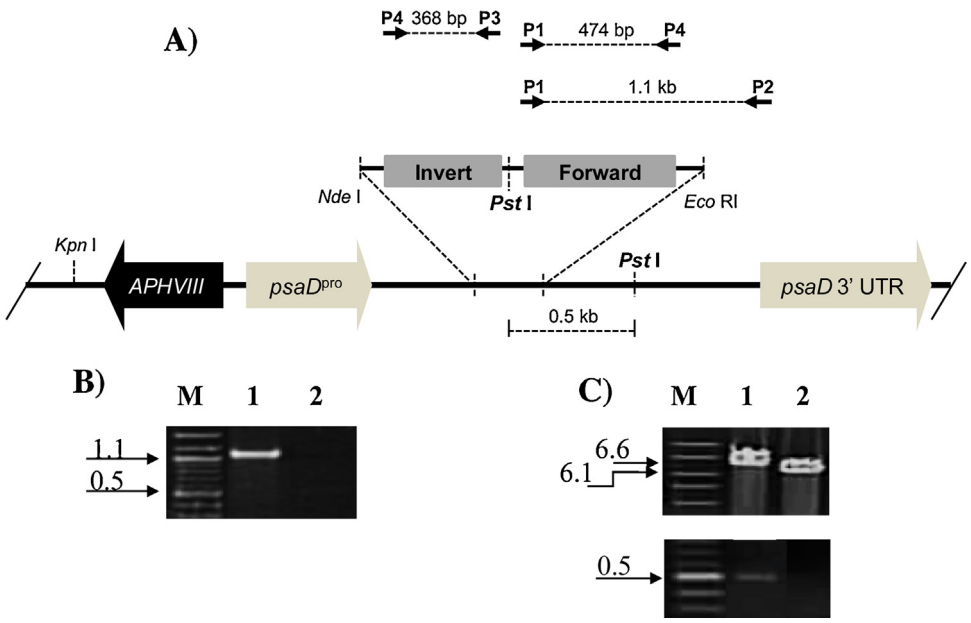

Fig. 1. (A) Schematic diagram of a 7.1-kb *C. reinhardtii* expression plasmid encoding dsRNA-YHV (pSL18-YHV). Hairpin-YHV cassette (1 kb) was inserted into a 6.1-kb pSL18, flanked by *psaD*^{pro} promoter and *psaD*3′UTR terminator at both ends, at *Eco* RI and *Nde* I restriction sites and a selective marker *APHVIII*. (B) PCR analysis using chimeric primers P1 (hairpin-forward) and P2 (pSL18). (C) Enzymatic digestion of the recombinant plasmid with *Pst* I. Lanes 1: pSL18-hpYHV; 2: pSL18 and M: 2 log DNA marker.

well-established for producing recombinant proteins (Rosales-Mendoza et al., 2012; Almaraz-Delgado et al., 2014). In particular, Kumar et al. (2013) demonstrated the successful transformation of *C. reinhardtii* chloroplast genome for expressing dsRNA targeting 3-hydroxykynurenine transaminase (3-HKT), the enzyme critical for the catabolism pathway of mosquitoes. Oral feeding of the microalgae expressing 3-HKT-dsRNA specifically induced significant larval mortalities (ranging from 30 to 50% depending on the transformant clones) relative to the control that received the non-transformant (15% mortality). The study suggested the possibility of developing *C. reinhardtii* for producing specific dsRNA that mediated inhibition of gene expression.

Here, we engineered the microalgae *C. reinhardtii* for expressing dsRNA specific to RNA dependent RNA polymerase (RdRp) of shrimp yellow head virus (YHV). The RdRp-sequence specific dsRNA was previously shown to inhibit YHV replication efficiently (Saksmerprome et al., 2009; Theerawanitchpan et al., 2012). The expression vector pSL18, originally used for protein expression in *C. reinhardtii* (Fischer and Rochaix, 2001), was constructed to encode hairpin dsRNA-YHV under the control of "*psaD*" promoter. Transformation of *C. reinhardtii* nuclear genome with pSL18-YHV was performed using glass bead method (Kindle, 1990). Quantitative RT-PCR was developed to detect dsRNA-YHV expression in *C. reinhardtii*. Finally, *C. reinhardtii* expressing dsRNA-YHV were subjected to oral administration assay to examine anti-YHV activity in post-larval shrimp.

2. Materials and methods

2.1. Microalgal strain and cultured condition

Non-transformed *C. reinhardtii* strain (CC-503 cw92 mt+) was obtained from Assistant Professor Dr. Kittisak Yokthongwattana, Department of Biochemistry, Mahidol University, Thailand. The *C. reinhardtii* was cultivated in TRIS acetate phosphate medium (TAP) on a rotary shaker (100 rpm) under continuous light (50 μmol photon m^{-2} s^{-1}) at 25 °C for 4 days until cell density of 2×10^6 cells/mL was obtained.

2.2. Construction of recombinant plasmid for microalgal expression of viral-specific dsRNA

C. reinhardtii nuclear expression vector pSL18 (Fischer and Rochaix, 2001) was constructed to contain a hairpin cassette for YHV-specific dsRNA production. The hairpin expression cassette targeting YHV was originally constructed in pGEM as described in Saksmerprome et al. (2009). pGEM-hpYHV was excised with *Eco* RI and *Nde* I to release a 1-kb hairpin fragment flanked by the restriction sites. To create restriction sites compatible with the released hairpin fragment, pSL18 was digested with *Eco* RI and *Nde* I. The hairpin YHV fragment was ligated downstream of the *psaD* promoter into the digested pSL18, and the resulting pSL18-YHV plasmid was subsequently transformed into *E. coli* DH5α. Colony PCR analysis using P1 and P2 primers (Table 1) was performed and

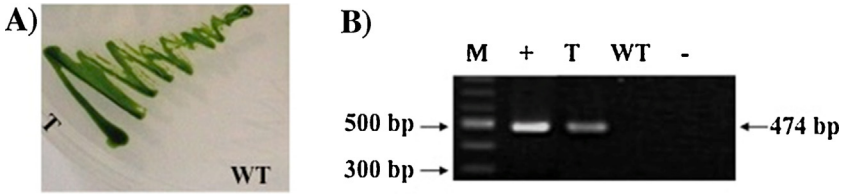

Fig. 2. (A) Growth of transformant (T) compared to non-transformant (WT) on a TAP agar plate with 10 μg/mL paromomycin after a biweekly sub-culture for at least a year. (B) PCR analysis with P1 and P4 primers. M, 2 log DNA marker; lanes +, pSL18-YHV; T, transformant; WT, non-transformed algae, and −; negative control (using water instead of DNA template).

Table 1
Primers used in this study and their nucleotide sequences.

Representative in diagram	Primer	Sequence (5′-3′)
P1	hpYHV_R[a]	TTGGGTTGAATCCTG
P2	M13_F[b]	GTAAAACGACGGCCAGT
P3	hpYHV_Rin[a]	GAATTCTAGCCATGC
P4	hpYHV_F[a]	GCATGTCCTGTTCTC
N/A	GY2[c]	CATCTGTCCAGAAGGCGTCTATGA
N/A	GY4[c]	CATCTGTCCAGAAGGCGTCTATGA
N/A	V_actin_F	CCTCGCTGGAGAAGTCCTAC
N/A	V_actin_R	TGGTC CAGACTCGTC GTACTC
N/A	CBLP_F[d]	GCCACACCGAGTGGGTGTCG
N/A	CBLP_R[d]	CCTTGCCGCCCGAGGCGCACAGCG

N/A; not applicable.
[a] Referred in Saksmerprome et al. (2009).
[b] Referred in Martinez-Garcia et al. (2012).
[c] Referred in Cowley et al. (2004).
[d] Referred in Eriksson et al. (2004).

the PCR-positive clone was subjected to restriction enzyme analysis with *Pst* I.

2.3. *Nuclear transformation of the microalgae* C. reinhardtii *by glass bead method*

Nuclear transformation by glass bead method was performed as previously described (Kindle, 1990). Briefly, 8 μg of linearized pSL18-YHV were added into 300 μL of concentrated 2×10^8 cell/mL in the presence of 300 mg glass beads (Sigma, USA). The reaction mixture was a vortex for 15 s, and was plated on a selective TAP medium supplemented with 10 μg/mL paromomycin. Transformed plates were incubated under dark condition for 16–24 h, and were continued to grow under light at 50 μmol photon m^{-2} s^{-1} for 1 week. Identification of transformants carrying hairpin YHV expression cassette was performed by colony PCR using P1 and P4 primers (Table 1). The cycling conditions composed of an initial denaturation step at 94 °C for 5 min, followed by 35 cycles at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s and extension step at 72 °C for 7 min and termination at 16 °C. To generate stable transformant, the positive clone was sub-cultured on a new selective agar every 2 weeks. To confirm stable integration of hairpin cassette in the nuclear genome, the positive transformant growing on the selective medium for at least one year was subjected to DNA extraction using 10 mM EDTA, pH 8.0 (Cao et al., 2009). PCR was performed as the above-mentioned colony PCR condition.

2.4. *Microalgal expression of dsRNA targeting YHV (dsRNA-YHV) and quantitation by qRT-PCR*

Total RNA was extracted from 100-mL (1×10^8 cells) cultures using TriPure Isolation Reagent (Roche, Germany), followed by

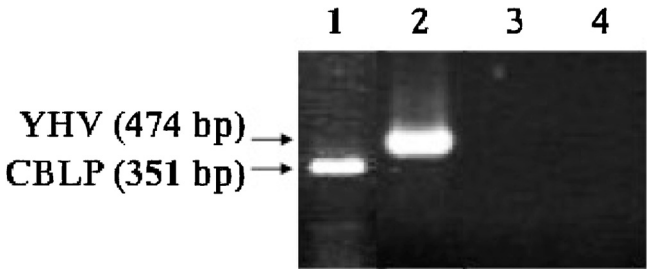

Fig. 3. PCR analyses on algal RNA extracts treated with DNase and RNaseA. Lanes 1, CBLP+, positive control for gDNA contamination; 2, positive control for YHV plasmid contamination; 3, reaction using CBLP-specific primers; 4, reaction using YHV-specific primers.

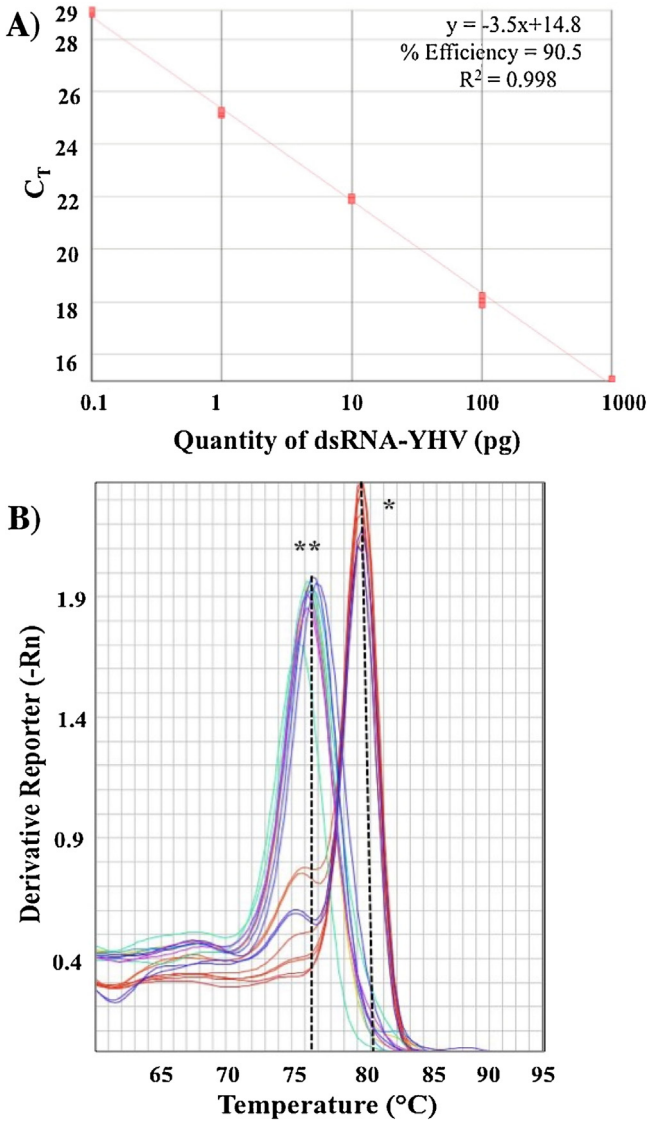

Fig. 4. Quantitation of microalgal dsRNA by qRT-PCR. (A) Strandard curve constructed with tenfold-diluted dsRNA-YHV expressed from *E. coli* HT115. (B) Derivative melting curve for standard curve samples (10-fold dilutions of bacterially-expressed dsRNA-YHV) and microalgal dsRNA-YHV. * indicated the melting temperature of the standards and microalgal dsRNA. ** indicated the melting temperature of negative control (without RNA template), RNA extracts from WT algae, RNaseIII-treated bacterial dsRNA-YHV, and RNaseIII-treated microalgal dsRNA-YHV.

DNase and RNaseA treatments. The treated RNA was then subjected to the following PCR and qRT-PCR analyses. To rule out possibility of genomic DNA and plasmid contamination, PCR was performed using (1) CBLP_F and CBLP_R primer pair that are specific for *C. reinhardtii* G-protein β-subunit-like polypeptide (CBLP); (2) P1 and P4 primer pair, respectively. Cycling conditions were the same as those for the colony PCR.

Twenty microliter of one-step qRT-PCR reaction contained 10 μL of KAPA SYBR® FAST qRT-PCR (Kapa Biosystems, USA) master mix, 6 μL of nuclease-free water, 200 nM of each P3 and P4 primers targeting hairpin, 200 μM of dUTP, 0.4 μL of ROX references dye, 2 μL of RNA template. Each reaction was performed in duplicate in an ABI 7500 Fast real-time PCR system (Applied Biosystems, USA). A series of 10-fold dilutions (0.1 pg–1 ng) of dsRNA-YHV expressed from *E. coli* HT115 were subjected to qRT-PCR to plot a standard curve. Quantity of dsRNA-YHV was determined from DNase- and RNaseA-treated RNA from 100-mL transformant culture. In parallel,

microalgal RNA treated with RNaseIII was also subjected to qRT-PCR as a control. Thermocycling conditions consisted of a 5 min at 42 °C for cDNA synthesis, 5 min at 95 °C for inactivate RT, 40 cycles of 10 s at 95 °C for cDNA template denaturation, 32 s at 60 °C for annealing and extension. A melting curve (60–95 °C) was generated for each assay.

2.5. Feeding trial

Non-transformed *C. reinhardtii* and its transformant cultures were centrifuged at 4500 rpm, 4 °C to obtain cell pellets. Each type of pellets was spread on shrimp feed (Syaqua Co., Ltd. Bangkok, Thailand) at 1×10^8 cells/g-feed using alpha starch as a binder. Specific pathogen free (SPF) *Penaeus vannamei* post-larvae (0.1 g) from Syaqua Co., Ltd. (Bangkok, Thailand) were divided into 4 groups (each had 2 replicates), and each group comprised 9 shrimp. Negative (no YHV infection) and positive controls were orally administered with the normal feed (no addition of algae) throughout the experiment. The two experimental groups received feed supplemented with non-transformed *C. reinhardtii* and its transformant expressing dsRNA-YHV. Feeding dose rate was at 5% of their body weight, and shrimp were fed 5 times a day. At day 5 of the experiment, a positive group and two experimental groups were challenged with YHV by oral feeding with the YHV-infected homogenized shrimp at 50% of their body weight. After YHV infection, a cumulative survival rate of each group was determined for 9 days. All data were calculated as a percentage of shrimp survival, and error bars represented standard error of the mean. Shrimp at the end of experiment were collected for RT-PCR analysis using YHV-specific primers (GY2 and GY4 in Table 1). In pararel, internal control actin gene (GenBank accession no. AF300705.2, nucletotides 775–1175) was amplified using V_actin_F and V_actin_R primers (Table 1). Both RT-PCR analyses of YHV and actin were performed as follows: 5 min at 50 °C, 5 min at 94 °C, 35 cycles of 10 s at 94 °C, 30 s at 59 °C and 30 s at 68 °C and extension at 68 °C for 5 min for 30 cycles.

3. Results and discussion

3.1. Recombinant plasmid pSL18-YHV was successfully constructed to contain YHV-specific hairpin expression cassette

The recombinant plasmid pSL18-YHV was generated by introducing a 1-kb hairpin expression cassette (hp-YHV) into a 6.1-kb microalgal expression vector pSL18, which was originally developed for an efficient recombinant protein production in *C. reinhardtii* (Fischer and Rochaix, 2001), downstream of *psaD* promoter (Fig. 1A). PCR analysis using a chimeric primer pair, P1 targeting hairpin cassette and P2 targeting pSL18, revealed the expected band of ~1.1 kb in Lane 1, Fig. 1B whereas no amplified product from pSL18 empty vector in Lane 2, Fig. 1B. In addition, *Pst* I digestion analysis on pSL18-YHV generated two fragments of 0.5-kb and 6.6-kb fragments of hairpin forward fragment and the rest of the digested pSL18-YHV, respectively (Fig. 1C, Lane 1), whereas an original pSL18 linearized by *Pst* I gave rise to a single 6.1-kb band (Fig. 1C, Lane 2). Both PCR and enzymatic digestion analyses indicated that the recombinant plasmids pSL18-YHV was successfully obtained for dsRNA expression in *C. reinhardtii*.

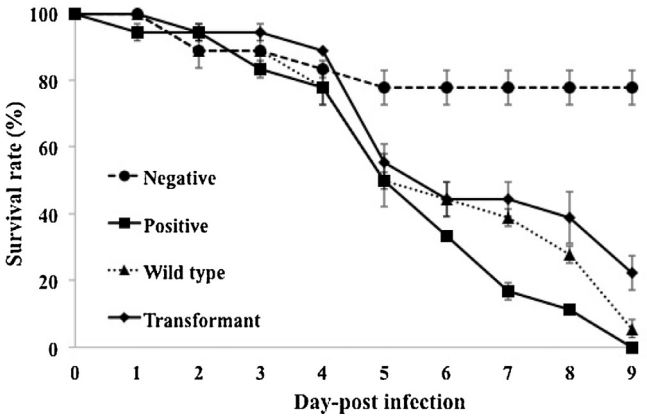

Fig. 5. Percentage of shrimp survival as a function of time after YHV challenge. Bars represent the means ± standard errors. Negative = normal feed and no YHV infection; positive = normal feed; wild-type = non-transformed algae; transformant = algae expressing dsRNA-YHV.

3.2. Transgenic microalga capable of producing YHV-specific dsRNA

Due to the presence of a selective marker *APHVIII* (paromomycin-resistant gene) on pSL18-YHV, *C. reinhardtii* strain transformed with pSL18-YHV was able to grow on TAP supplemented with 10 μg/mL paromomycin, and the integration of hairpin cassette in the algal genome was confirmed by PCR using YHV-specific P1 and P4 primers. Stable transformant was selected by its survival after sub-culturing every 2 weeks for at least a year. It appeared greenish on the selective media after 1-week

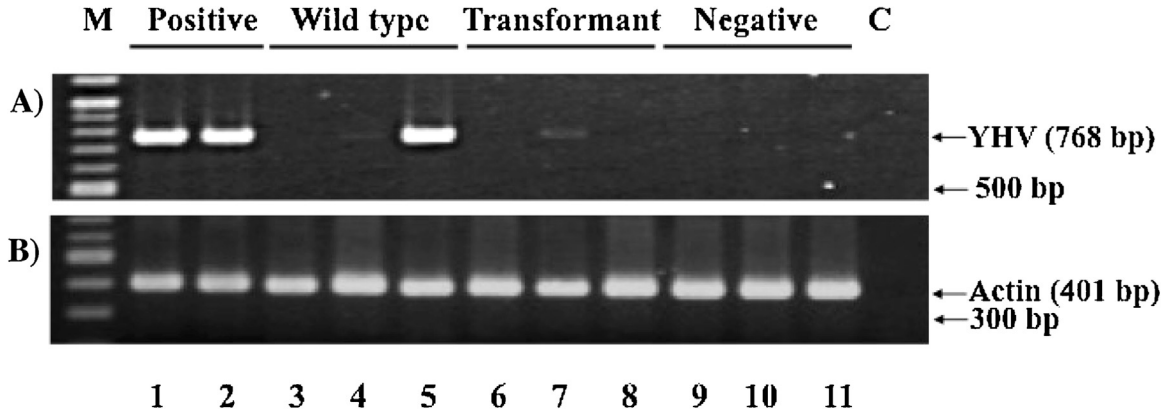

Fig. 6. YHV-specific suppression by *C. reinhardtii* expressing dsRNA-YHV in the shrimp at 9 dpi. (A) RT-PCR using YHV-specific primers on three individuals without YHV infection (lanes 9–11), on those fed with non-transformed algae (lanes 3–5), on those fed with transformed algae (lanes 6–8), and on two individuals YHV-positive control group (lanes 1-2). (B) RT-PCR using shrimp actin-specific primers were performed in parallel as internal control, C; negative control (using water instead of RNA template), M; 2 log DNA ladder.

incubation at 25 °C, whereas the non-transformed cell did not grow (Fig. 2A). PCR followed by gel electrophoresis of the selected transformant revealed the 474-bp YHV-targeted amplicon (Fig. 2B, Lane T), suggesting stable integration of hairpin YHV cassette in the transgenic *C. reinhardtii*.

Total RNA extracted from 100-mL culture (~1×10^8 cells) during the log phase was treated with DNase and RNaseA to remove any DNA and single-stranded RNA, respectively. This treated algal RNA was subjected to PCR and qRT-PCR analyses. Using the algal RNA as template, PCR with primers specific for microalgal house-keeping gene and for hairpin cassette were performed to rule out any possibility of DNA contamination in the RNA extract. Positive controls for genomic DNA and plasmid contamination were the reactions with plasmids containing CBLP gene and YHV hairpin cassette, respectively (Fig. 3 Lanes 1–2). No amplified product was obtained from the reaction with each set of primers (Fig. 3, Lanes 3–4), indicating that the extracted RNA was not contaminated by either genomic DNA or the recombinant plasmid during RNA extraction.

For qRT-PCR, a standard curve was plotted using tenfold-diluted dsRNA-YHV expressed from *E. coli* HT115. The bacterial dsRNA stock was quantitated, prior to the assay, by measuring UV absorbance at 260 nm, and dsRNA concentration was calculated in μg/μL. The regression equation between the bacterial dsRNA-YHV and Ct is $y = -3.5x + 14.8$ ($R^2 = 0.998$), where x and y represent the quantity of dsRNA-YHV in picograms and the Ct value, respectively (Fig. 4A). The efficiency of the reaction (E) was calculated to be 90.5% by using the formula $\%E = (10^{-\frac{1}{m}} - 1) \times 100$, m = slope of 1001/of the standard curve. The amount of microalgal dsRNA from 100-mL culture (~1×10^8 cells) was 41.0 ± 2 ng. Melt-curve analysis suggested that the product amplified from *C. reinhardtii* dsRNA was the same as that of *E. coli* dsRNA-YHV, with the melting range (Tm) of 81.7 ± 0.1 °C as indicated by a single asterisk on Fig. 4B. In contrast, total RNA from wild-type algae should not contain any dsRNA-YHV, and therefore its qRT-PCR should not yield an amplicon with the above-mentioned Tm. Non-specific amplification from wild-type RNA resulted in the amplicons that melted at temperatures below that of the desired product as indicated by a double-asterisk on Fig. 4B. Similar melting range ($Tm = 77.4 \pm 0.4$ °C) was observed in the negative control reaction without RNA template, and those with RNaseIII-treated dsRNA-YHV from *E. coli* and transgenic *C. reinhardtii*. The non-specific amplifications in the reactions without RNA template and those with RNase-III treated dsRNA would more likely be the results of primer-dimers and short dsRNAs (RNaseIII-cleavage product) that would melt at lower temperatures than the desired product from dsRNA-YHV.

The melt-curve analysis confirmed that the amplified signals are the results of the desired dsRNA, not other non-specific dsRNA. The fact that RNAi machinery was identified in the microalgal nucleus (Casas-Mollano et al., 2008) suggested that majority of long dsRNA molecules synthesized in the nucleus would be constantly processed into short dsRNAs of 19–21 nt. Therefore, the amount of dsRNA-YHV by the method developed herein would more likely represent the remaining long dsRNA-YHV prior to the cleavage reaction.

3.3. *Feed formulated with* C. reinhardtii *expressing dsRNA-YHV enhanced shrimp survival after YHV infection*

At 9-day post YHV infection (dpi.), the group receiving the microalgae expressing dsRNA-YHV at 1×10^8 cells/g-feed had a higher survival rate than the group fed with non-transformed algae (22 *vs.* 5%), whereas all animals receiving the normal feed (no algae) were dead (Fig. 5). RT-PCR analysis revealed that two of three survived shrimp from the group fed with the algae expressing dsRNA-YHV remained free from YHV at 9 dpi. (Fig. 6, Lanes 6 and 8). It is, therefore, convincing that algae expressing dsRNA-YHV enhanced shrimp survival by specific YHV inhibition. In contrast, non-transformed algae did not appear to protect shrimp against the virus, and two of three survived shrimps from this group at 9 dpi. were found to be YHV-infected (Fig. 6, Lanes 4 and 5) as well as two shrimps from YHV-positive group (Fig. 6. Lanes 1 and 2)

Bioengineering *C. reinhardtii* by nuclear transformation with the developed expression vector (pSL18-dsRNA) was shown to successfully produce dsRNA targeting a virus, and the microalgal growth culture can be easily scaled up for future commercial application in shrimp aquaculture. This study also demonstrated that oral application of *C. reinhardtii* expressing dsRNA targeting the lethal YHV provided specific viral inhibition in shrimp, although magnitude of antiviral efficiency has yet to be optimized by several means in the future such as varying dsRNA dose in the formulated feed. In our knowledge, this is the first report on production of antiviral dsRNA using the microalgae *C. reinhardtii* for shrimp disease control. Given that viral target sequence is available, the dsRNA expression system in *C. reinhardtii* should be applicable to combat with any virus of interest.

Acknowledgments

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